Published in final edited form as:
Transgenic Res. 2010 August ; 19(4): 611–620. doi:10.1007/s11248-009-9345-1.

Optimization of square-wave electroporation for transfection of porcine fetal fibroblasts

Jason W. Ross[1,4], **Jeffrey J. Whyte**[2], **Jianguo Zhao**[1,3], **Melissa Samuel**[1,3], **Kevin D. Wells**[1], and **Randall S. Prather**[1,3]

[1]Division of Animal Sciences, University of Missouri, Columbia, MO 65211

[2]Department of Biomedical Sciences, University of Missouri, Columbia, MO 65211

[3]National Swine Resource and Research Center, University of Missouri, Columbia, MO 65211

Abstract

Development of a transgenic porcine biomedical research model requires effective delivery of DNA into the donor cell followed by selection of genetically modified somatic cell lines to be used for nuclear transfer. The objective of the current study was two-fold: 1) to compare the effectiveness of a single 1 ms pulse of different voltages (V; 100, 150, 200, 250, 300, 350) and multiple 1 ms pulses (1, 2, 3, 4 or 5) at 300 V for delivery and expression of super-coiled GFP vector in surviving cells of three fetal fibroblast cell lines, and 2) to determine the ability of these electroporation parameters to produce stably transfected fibroblast colonies following G418 selection. Cell line ($P < 0.001$) and voltage ($P < 0.001$) affected DNA delivery into the cell as assessed by GFP expression while survival at 24 hrs was affected by voltage ($P < 0.001$) and not by cell line ($P = 0.797$). Using a single pulse while increasing voltage resulted in the percentage of GFP expressing cells increasing from 3.2 ± 0.8% to 43.0 ± 3.4% while survival decreased from 90.5 ± 8.0% to 44.8 ± 2.0%. The number of pulses at 300V significantly affected survival ($P < 0.001$) and GFP expression ($P < 0.001$). Survival steadily decreased following 1 to 5 pulses from 63.2 ± 6.3% to 3.0 ± 0.3% with GFP expression of surviving cells increasing from 35.6 ± 2.67% to 71.4 ± 6.1%. Electroporation of a selectable marker at a 1:1 copy number ratio to a coelectroporated transgene resulted in 83% of G418 resistant colonies also being PCR positive for the secondary transgene. These electroporation conditions, specifically, three 1 ms pulses of 300 V to 200 μL of 1 $\times 10^6$ cells/mL in the presence of 12.5 μg DNA/mL effectively introduced DNA into somatic cells. The utilization of these conditions produced numerous transgenic fibroblast colonies following G418 selection that when used for somatic cell nuclear transfer resulted in the production of live offspring.

Introduction

Several methods have been established that enable the production of transgenic livestock including pronuclear injection, intracytoplasmic sperm injection (ICSI), sperm-mediated gene transfer and somatic cell nuclear transfer (SCNT). These methodologies, each having advantages, have all been utilized to create transgenic large animals. In recent years, SCNT has primarily been used due to several advantages such as the lack of mosaic transgene integration (germline transmission is always achieved) and SCNT is the only method to knock out (KO) a gene in domestic animals (see review) (Ross et al. 2009a) The efficiency of SCNT to create transgenic clones relies heavily on the ability to make genetic modifications to the somatic cell genome prior to nuclear transfer. Numerous types of somatic cells have been

[4]Current Address: 2356E Kildee Hall, Iowa State University, Ames, Iowa 50011

utilized to create transgenic pigs, such as fetal somatic stem cells (Hornen et al. 2007), salivary gland-derived progenitor cells (Kurome et al. 2008), pre-adipocytes (Tomii et al. 2005), adult fibroblast (Beebe et al. 2007; Brunetti et al. 2008) and fetal fibroblasts (Hyun et al. 2003; Lai et al. 2002a; Lai et al. 2002b; Park et al. 2001). Fetal fibroblasts have become the most used somatic cell for the production of transgenic livestock because they are easily collected and cultured, capable of being genetically modified, and posses the ability to produce live offspring. In addition to the reproducible ability of making cloned pigs, porcine fetal fibroblasts have a doubling time and life span that make them suitable for genetic modification the utilization of selectable markers such as Geneticin (G418).

Numerous strategies have been utilized to introduce exogenous DNA into porcine fetal fibroblasts, including lipid based delivery (Hyun et al. 2003; Lee et al. 2005), viral delivery (Lai et al. 2002a; Rogers et al. 2008), and electroporation (Dai et al. 2002; Ramsoondar et al. 2003; Watanabe et al. 2005). While each of these methods has been utilized to successfully produce transgenic piglets, optimal conditions for each of these strategies capable of producing predictable results with respect to exogenous DNA delivery into the cytoplasm of porcine fetal fibroblast have not been described.

The objective of this study was to establish an approach that identifies optimal parameters for electroporation of exogenous DNA into fetal fibroblast and eventual selection of transgenic fetal fibroblasts colonies to be used for the production of genetically modified cloned pigs. Herein, we described the comparison of these experimental conditions for fetal fibroblast from 3 different genetic backgrounds. We then expanded the analysis to demonstrate effective methods to produce randomly integrated transgenic cell lines for utilization in SCNT programs. This approach has allowed the ability to effectively produce fetal fibroblasts lines that are predictably transgenic and capable of producing cloned offspring.

Materials and Methods

Fetal Fibroblast Collection

Fetal fibroblasts were collected as described (Lai and Prather 2003) with minor modifications. Minced tissue from each fetus was digested in 20 mL of digestion media (DMEM supplemented with 200 units/ mL collagenase and 25 Kunitz units/mL DNaseI) for 5 hrs at 38.5 °C. Digested cells were washed with DMEM, 15% fetal bovine serum (FBS) with 10 μg/mL gentamicin, cultured overnight, then collected and frozen at −80 °C in aliquots in FBS with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

Cell Lines, Culture Conditions and Electroporation

Three different fetal fibroblast cell lines of different genetic backgrounds (L: Landrace, Y: Yucatan, N: NIH miniature [CC haplotype]) of similar passage number (2–6) were cultured overnight and grown to 75–85% confluency in Dulbeco's Modified Eagles Medium containing L-glutamine and 1 g/L D-glucose; DMEM) supplemented with 15% fetal bovine serum, 2.5 ng/mL basic fibroblast growth factor (Sigma) and 10 μg/mL gentamicin. Media was changed 4 hours prior to transfection. Fibroblast cells were washed for 1–2 min with phosphate buffered saline (PBS; Invitrogen) and digested with 0.05% trypsin-EDTA (Invitrogen; 1 mL per 75 cm^2). As soon as cells detached, they were resuspended in DMEM, pelleted at 600 × g for 10 min, resuspended in 10 mL Opti-MEM (Invitrogen), and then quantified by using a hemocytometer and repelleted. Cells were resuspended in transfection media (75% cytosalts [120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4; pH 7.6, 5 mM $MgCl_2$] (van den Hoff et al. 1992) and 25% Opti-MEM) quantified again and the cell concentration was adjusted to 1×10^6 cells/mL. All electroporations consisted of 3.93×10^{11} DNA molecules per transfection

in 200 μL transfection media containing 1×10^6 cells/mL. Each electroporation utilized square-wave pulses administered through a BTX ECM 2001 in 2 mm gap cuvettes.

Exogenous DNA Constructs

Transient transfection assays were conducted with supercoiled pCAGG-EGFP (Whitworth et al. 2009) that expresses cytoplasmic localized green fluorescent protein. For stable transfections, Fsp1 was utilized to cut phEFnGFP (Wells and Powell 2000) to produce a linearized DNA fragment containing the human elongation factor 1 promoter to drive green fluorescent protein expression with two nuclear localization signal peptides from SV40 large T antigen. Digestion with FspI followed by gel purification of the linearized fragment was also essential for the removal of the functional neomycin cassette. For transfections that were followed by selection, an 1831 bp linearized fragment containing the phosphoglycerol kinase promoter and polyadenylation signal flanking the neomycin resistance peptide coding sequence was used.

Voltage and pulse number to optimize maximize delivery of exogenous DNA into different porcine fetal fibroblast cell lines

Experiment 1—To examination of the effect of voltage on the delivery of supercoiled pCAGG-EGFP, 3.93×10^{11} molecules (2.5 μg) of purified vector DNA was mixed into 200 μL of transfection media containing 1×10^6 cells/mL. A single 1 ms pulse of 0, 100, 150, 200, 250, 300 and 350 V was administered to each of 3 different cell lines (3 reps/voltage/cell line). Following electroporation, the cells were resuspended in 10 mL cell culture media and 5 mL was plated into a single well of a 6-well culture dish. Twenty-four hours after transfection, each well was washed with PBS and fresh media containing Hoechst 33258 was added and allowed to stain the nuclei for 20 min at 38.5°C. For each well, using a 20× objective on a Nikon Diaphot equipped with an ultraviolet lamp and appropriate filters, the total number of cells based on Hoechst nuclear staining and the percentage expressing EGFP were determined in a field of view (five fields of view per well were averaged). Survival was determined as the number of cells in the average field of view divided by the number of cells in the 0 voltage group. The percentage green is determined by dividing the number of fluorescent green cells by the number of total nuclei within an averaged field of view.

Experiment 2—Based on the results of a single pulse at multiple voltages, 300 volts was used to examine the effect of multiple pulses. At 300 V, 0, 1, 2, 3, 4 or 5 pulses (1 ms each) were administered to each of the different cell lines (3 reps/pulse number/cell line) and the cells were resuspended, plated, and examined at 24 hours as described for experiment 1.

Identification of optimal voltage for production of neomycin resistant colonies with the stable integration of a co-transfected transgene

While greater voltage used for 3 pulses increased the number of cells transiently transfected, it also compromised the health of surviving cells. This experiment was designed with three purposes: 1) to identify the voltage for which 3 pulses produces the most stably transgenic colonies following G418 (400 μg/mL) selection, 2) to identify the co-integration rate when electroporating two separate linearized constructs followed by G418 selection and 3) to determine the effect of reducing the copy number of selectable marker genes while maintaining a consistent number of free DNA ends on G418 colony production. Using the methods as described, we tested the effects of 3 pulses at 0, 100, 150, 200, 250 and 300 V on the ability to produce stably transfected fetal fibroblasts and the ratio of the selectable marker copies to secondary transgene copy number (1:1, 1:3 and 1:9) on co-integration rate. Each transfection (n = 3/voltage/transgene ratio) utilized a consistent number of molecules (3.93×10^{11}). Following electroporation, NIH miniature pig fetal fibroblast cells were diluted in cell culture

media and plated in 100 mm tissue culture dishes at a density of 24000, 4800 and 900 cells per dish based on the cell density prior to electroporation (n = 3 dishes/density/electroporation). Additionally, for each electroporation (n =15; 5 voltages repeated over 3 DNA ratios), 40 and 200 cells were plated per well of a 48-well dish (one dish per stocking density) to produce individual G418 resistant colonies representing each combination of DNA ratio and voltage.

Thirty-six hours following the electroporation, media was changed to apply G418 (400 μg/mL). G418 selection was maintained for 14 days, changing the media every 4–5 days. Following selection, colonies present in 100 mm plates were rinsed with MEM then incubated in 6 mL of Coomassie Blue R250 (Teknova, Hollister, CA) for 5 min at RT followed rinsing thoroughly with tap water. The number of confluent colonies for each voltage replication were determined by visually inspecting each dish and determining the average of the three dishes for each stocking selectable marker amount, voltage and cell stocking density. To statistically determine the effect of voltage and selectable marker copy number the data were normalized by conversion to a percentage of the lowest treatment. To evaluate the effect of selectable marker copy number, the data for each voltage were converted to a percentage of colonies formed respective to the lowest voltage for each selectable marker amount. To evaluate the effect of voltage on the number of colonies formed, the data for each selectable marker amount were converted to a percentage of the number of colonies formed respective to the lowest selectable marker amount. These data conversions were possible because the number of colonies was consistently lowest in the 100 V group and the lowest selectable marker copy number (3.93×10^{10}) group.

To determine the integration frequency of the secondary GFP coding transgene, individual colonies selected from single wells of a 48-well dish were harvested via 0.05% trypsin-EDTA and centrifuged at maximum speed for 5 min. Following centrifugation, the cell pellet was resuspended in 20 μL lysis buffer (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.4 mg/mL proteinase K), incubated at 65°C for 15 min for cell lysis followed by 95°C for 10 min to inactivate proteinase K; similar to previously described colony preparation (Dai et al. 2002). Two μL of the lysed cell solution was added per 25 μL PCR reactions to detect the presence of an endogenous genomic region (forward primer 5'-ACGTCCTTGTAGGCACAGCTAACA-;3'; reverse primer 5'-GTCACTGTTTGTTTGGTGCCGTGA-3'), the coding region of the neomycin resistance gene (forward primer 5'-ATGGATACTTTCTCGGCAGGAGCA-3'; reverse primer 5'-ACAAGATGGATTGCACGCAGGTTC-3'), and the presence of the GFP coding sequence (forward primer 5'-TGACCCTGAAGTTCATCTGCACCA-;3'; reverse primer 5'-TTACTTGTACAGCTCGTCCATGCC-3'). Expression frequency of the EF1 promoter with the nuclear localized GFP was determined via visualization for GFP expression via UV illumination and following staining with Hoechst dye.

Production of transgenic pigs utilizing the identified electroporation parameters

Fetal fibroblast cells were co-transfected with a 17.5 kb transgene and the 1831 bp neomycin resistant selectable marker at a 10:1 molar ratio using three 1 ms pulses of 250 V in cytosalts. Cells underwent selection with G418 as described above for 14 days. Fibroblast cells from clonal colonies and/or pools of G418 resistant colonies were utilized for somatic cell nuclear transfer followed by embryo transfer using procedures previously described for our lab (Lai and Prather 2003; Zhao et al. 2009) coupled with two different embryo fusion/activation methods, NT1(electrical fusion and activation) and NT3 (electrical fusion and chemical activation), as described (Whitworth et al. 2009).

Statistical Analysis

Prior to statistical analysis, all percentage data were arcsine transformed. Following transformation, the analysis of the transient transfection experiment tested the effects of voltage, cell line, pulse number and appropriate interactions on the survival and percentage of cells expressing GFP. For the analysis of stably transfected colonies, the effect of voltage, selectable marker copy number and their interaction was determined also using the MIXED procedure of SAS (Cary, NC). Significance ($P < 0.05$) was determined by using the probability differences of least squares means. Data are presented as arithmetic means ± SEM.

Results

Voltage and pulse number to optimize maximize delivery of exogenous supercoiled vector DNA into different porcine fetal fibroblast cell lines

Using the pCAGG promoter driving enhanced GFP (EGFP) expression, the number of transiently transfected cells was quantified by examining EGFP expression in the cytoplasm of cells (Figure 1a–c). The percent of cells transfected as determined by EGFP expression was affected by both cell line ($P < 0.001$) and voltage ($P < 0.001$) while no interaction between the two effects was observed ($P = 0.156$). Lines L and N were not different whereas line Y produced fewer transfected cells than either Line L or N ($P < 0.001$). Across multiple voltages, cell survival was not affected by a cell line ×voltage interaction ($P = 0.92$) or by cell line alone ($P = 0.47$), although it was greatly affected by the voltage ($P < 0.001$). Cell survival decreased steadily as single pulses of voltage increased with lowest survival occurring at 350 V.

Accounting for both cell survival and the percentage expressing EGFP, 300 V was determined to produce the greatest number of transiently transfected cells. For that reason we tested the effect of multiple pulses at a constant voltage (300 V). A cell line × pulse number effect was not detected for either GFP expression or survival. Keeping voltage consistent (300 V), the number of pulses significantly affected both survival ($P < 0.001$) and the percentage GFP expressing cells ($P < 0.001$). The percentage of transiently transfected cells increased with each pulse from 36 to 71% (1 to 5 pulses, average of all cell lines) with no significant difference being detected between 3, 4 or 5 pulses (Figure 2). Cell survival dramatically decreased from 63% to 3% for 1 to 5 pulses, with each pulse significantly ($P < 0.05$) reducing survival except for 4 and 5 pulses, which were not different.

Identification of optimal voltage for production of neomycin resistant colonies with the stable integration of a co-transfected transgene

The addition of 400 μg/mL of G418 to the cell culture media 36 hrs after transfection for 14 days was sufficient to produce colonies of approximately 12–18 mm in diameter (Figure 3a). The utilization of the EF1 promoter for expression of a nuclear localized GFP worked well in fibroblasts leading to expression in the majority of G418 resistant colonies, regardless of DNA ratio. While nuclear specific GFP was present in the majority of the colonies produced, its expression was always variegated within colonies (Figure 3b–d).

After correcting for the differences in selectable marker copy number by transforming the data as described, a significant ($P < 0.001$) effect of voltage on the number of colonies was detected (Figure 3e). It was determined that 100, 150 and 200 V were not significantly different from each other although 250 and 300 V produced significantly greater number of G418 resistant colonies than 100, 150 and 200 V. Keeping the number of total DNA molecules and free ends consistent, the number of selectable marker gene copies had a significant ($P < 0.001$) effect on the number of colonies produced per electroporation (Figure 3f); 1.97×10^{11} copies of the

selectable marker produced the greatest number of colonies compared to 9.83×10^{10} or 3.93×10^{10} ($P < 0.05$).

Cells from each electroporation were seeded in 48-well dishes at a stocking rate of 200 and 48 cells per well. In all, we were able to isolate 68, 20 and 6 individual colonies for the 1.97×10^{11} (1:1 ratio of selectable marker to transgene), 9.83×10^{10} (1:3 ratio) and 3.93×10^{10} (1:9 ratio) copies of the neomycin resistant gene, respectively. Visual examination revealed that 51 of 94 (54%) of all colonies expressing the co-transfected protein, also demonstrated variegated expression of nuclear-localized GFP (Figure 3b–d). Of the colonies verified via PCR to possess the neomycin coding region following G418 selection, 82% (56/68), 82% (14/17) and 80% (4/5) for the 1:1, 1:3 and 1:9 ratios of selectable marker to co-transfected transgene, respectively, also possessed the presence of a second gene (GFP), verified via PCR, when using G418 selection.

Electroporation conditions are conducive for the production of transgenic cloned pigs

Transgenic cells created using conditions described in this manuscript were utilized for five separate SCNT attempts followed by embryo transfer into surrogates. Four of these attempts established pregnancies in surrogates of which all went to term gestation. These four pregnancies resulted in the natural birth or cesarean delivery of 8 live piglets, of which 4 died shortly after delivery (Table 2). All piglets tested PCR positive for the transgene of interest (data not shown).

Discussion

Production of clonal somatic cell lines used for nuclear transfer ensures the presence of the transgene is ubiquitous in every cell's genome and subsequent germline transmission. A requirement for the production of genetically modified pigs through SCNT is the ability to effectively introduce foreign DNA into donor cells that are designated for the genetic modification and the reconstruction of embryos to derive piglets. Another prerequisite, particularly for the stable integration of a transgene is the ability to produce colonies that are consistently integrated with a gene of interest when co-transfected with a selectable marker. To this end, we have described an approach that leads to the identification of optimal electroporation conditions that successfully introduce exogenous DNA into the cytoplasm of porcine fetal fibroblasts. In addition, the described conditions produce sufficient, stably integrated transgenic cell lines for the production of genetically modified pigs following SCNT.

The conditions reported in this manuscript, specifically, three 1 ms pulses of 300 V to 200 μL (1×10^6 cells/mL in the presence of 12.5 μg DNA/mL) in a 2 mm cuvette consistently delivered exogenous DNA into the cytoplasm of 65–80% of surviving cells. Additionally, the utilization of these conditions were capable of producing sufficient colonies when transfecting a selectable marker followed by 2 weeks of selection in G418 (400 μg/mL). These conditions have been used and have resulted in the production of healthy, viable transgenic piglets (Ross et al. 2009b).

We have demonstrated that the utilization of supercoiled GFP vector DNA is a reliable and consistent method by which to identify the ability of electroporation conditions to deliver DNA. Additionally, the variation in this method was low enough that statistical differences between conditions could effectively be identified. The effectiveness of the multiple cells from different genetic backgrounds is important in that these conditions should be capable of being expanded to other lines of fetal fibroblasts.

While we did not directly compare square wave electroporation to the exponential decay pulse, the utilization of square-wave pulses produced conditions that consistently delivered

exogenous DNA into the cytoplasm of fetal fibroblasts. It has recently been demonstrated that the Amaxa Nucleofection System™ was capable of introducing exogenous DNA into 79% of surviving cells as determined by green fluorescent protein expression (Nakayama et al. 2007). These authors were capable of transiently transfecting 53% of surviving cells through electroporation, about 20–25% less than the best conditions reported in this data set using electroporation.

We observed a significant reduction in the number of G418 resistant colonies in response to reduced copy numbers of the PGK-Neo linearized construct, despite maintaining a consistent number of DNA ends per reaction (Figure 3f) by adjusting the number of copies of the EF1-GFP construct. These data confirm the notion that the number of copies of the construct containing the selectable marker is more important than the total number of DNA molecules, and demonstrates that there is a minimal copy number of selectable marker construct that should be used (Table 1, Figure 3f).

Fluorescence in situ hybridization indicates that in murine LTA fibroblasts, transgene integration did not favor specific chromosomal morphology or position within a chromosome, and integrations, regardless of copy number, occur within a single location greater than 90% of the documented integration events (Dellaire and Chartrand 1998). This finding, coupled with the data reported here, justifies co-electroporation of genes rather than investing in efforts engineering the selectable marker and the transgene into one construct, in that approximately 80% of the integration events possessing the selectable marker will contain the transgene when the ratio is 1:1.

The ability to create transgenic pigs, as well as other transgenic large animals through somatic cell nuclear transfer will be hastened when modifications to the somatic cell genome are capable of being accomplished through quick and effective strategies. The strategies reported here are capable of producing numerous transgenic cell lines that consistently possess the transgene of interest and when utilized for somatic cell nuclear transfer are capable of producing live transgenic pigs.

Acknowledgments

This work was supported in part by the National Institutes of Health National Center for Research Resources U42 RR018877 and Food for the 21st Century at the University of Missouri.

References and Literature Cited

Beebe L, McIlfatrick S, Grupen C, Boquest A, Harrison S, Faast R, Ashman R, Wengle J, Hamilton H, Nottle M. A comparison of two in vitro maturation media for use with adult porcine oocytes for adult somatic cell nuclear transfer. Cloning Stem Cells 2007;9(4):564–570. [PubMed: 18154516]

Brunetti D, Perota A, Lagutina I, Colleoni S, Duchi R, Calabrese F, Seveso M, Cozzi E, Lazzari G, Lucchini F, Galli C. Transgene expression of green fluorescent protein and germ line transmission in cloned pigs derived from in vitro transfected adult fibroblasts. Cloning Stem Cells 2008;10(4):409–419. [PubMed: 18823265]

Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol 2002;20(3):251–255. [PubMed: 11875425]

Dellaire G, Chartrand P. Direct evidence that transgene integration is random in murine cells, implying that naturally occurring double-strand breaks may be distributed similarly within the genome. Radiat Res 1998;149(4):325–329. [PubMed: 9525495]

Hornen N, Kues WA, Carnwath JW, Lucas-Hahn A, Petersen B, Hassel P, Niemann H. Production of viable pigs from fetal somatic stem cells. Cloning Stem Cells 2007;9(3):364–373. [PubMed: 17907947]

Hyun S, Lee G, Kim D, Kim H, Lee S, Nam D, Jeong Y, Kim S, Yeom S, Kang S, Han J, Lee B, Hwang W. Production of nuclear transfer-derived piglets using porcine fetal fibroblasts transfected with the enhanced green fluorescent protein. Biol Reprod 2003;69(3):1060–1068. [PubMed: 12773429]

Kurome M, Tomii R, Ueno S, Hiruma K, Matsumoto S, Okumura K, Nakamura K, Matsumoto M, Kaji Y, Endo F, Nagashima H. Production of cloned pigs from salivary gland-derived progenitor cells. Cloning Stem Cells 2008;10(2):277–286. [PubMed: 18373475]

Lai L, Park KW, Cheong HT, Kuhholzer B, Samuel M, Bonk A, Im GS, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells. Mol Reprod Dev 2002a;62(3): 300–306. [PubMed: 12112592]

Lai L, Prather RS. Production of cloned pigs by using somatic cells as donors. Cloning Stem Cells 2003;5 (4):233–241. [PubMed: 14733743]

Lai LX, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 2002b;295(5557):1089–1092. [PubMed: 11778012]

Lee SL, Ock SA, Yoo JG, Kumar BM, Choe SY, Rho GJ. Efficiency of gene transfection into donor cells for nuclear transfer of bovine embryos. Mol Reprod Dev 2005;72(2):191–200. [PubMed: 15971253]

Nakayama A, Sato M, Shinohara M, Matsubara S, Yokomine T, Akasaka E, Yoshida M, Takao S. Efficient transfection of primarily cultured porcine embryonic fibroblasts using the Amaxa Nucleofection system. Cloning Stem Cells 2007;9(4):523–534. [PubMed: 18154513]

Park KW, Cheong HT, Lai LX, Im GS, Kuhholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Animal Biotechnology 2001;12(2):173–181. [PubMed: 11808633]

Ramsoondar JJ, Machaty Z, Costa C, Williams BL, Fodor WL, Bondioli KR. Production of alpha 1,3-galactosyltransferase-knockout cloned pigs expressing human alpha 1,2-fucosylosyltransferase. Biol Reprod 2003;69(2):437–445. [PubMed: 12672664]

Rogers CS, Hao Y, Roklina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS. Production of CFTR-null and CFTR-deltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. Journal of Clinical Investigation 2008;118(4):1571–1577. [PubMed: 18324337]

Ross JW, Prather RS, Whyte JJ, Zhao J. Cloning and transgenics: progress and new approaches in domestic animals. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2009a;4(38):13.

Ross, JW.; Zhao, J.; Walters, EM.; Samuel, M.; Narfstrom, K.; Jeong, M.; DeMarco, MA.; McCall, MA.; Kaplan, HJ.; Prather, RS. Somatic cell nuclear transfer to create a miniature swine model of retinitis pigmentosa. New Orleans, LA: Apr 18–22. 2009b 2009

Tomii R, Kurome M, Ochiai T, Wako N, Ueda H, Hirakawa K, Kano K, Nagashima H. Production of cloned pigs by nuclear transfer of preadipocytes established from adult mature adipocytes. Cloning Stem Cells 2005;7(4):279–288. [PubMed: 16390264]

van den Hoff MJ, Moorman AF, Lamers WH. Electroporation in `intracellular' buffer increases cell survival. Nucleic Acids Res 1992;20(11):2902. [PubMed: 1614888]

Watanabe S, Iwamoto M, Suzuki S, Fuchimoto D, Honma D, Nagai T, Hashimoto M, Yazaki S, Sato M, Onishi A. A novel method for the production of transgenic cloned pigs: electroporation-mediated gene transfer to non-cultured cells and subsequent selection with puromycin. Biol Reprod 2005;72 (2):309–315. [PubMed: 15385422]

Wells KD, Powell AM. Blastomeres from somatic cell nuclear transfer embryos are not allocated randomly in chimeric blastocysts. Cloning 2000;2(1):9–22. [PubMed: 16218842]

Whitworth KM, Li R, Spate LD, Wax DM, Rieke A, Whyte JJ, Manandhar G, Sutovsky M, Green JA, Sutovsky P, Prather RS. Method of oocyte activation affects cloning efficiencyin pigs. Mol Reprod Dev 2009;76(5):490–500. [PubMed: 19090011]

Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS. Significant Improvement in Cloning Efficiency of an Inbred Miniature Pig by Histone Deacetylase Inhibitor Treatment After Somatic Cell Nuclear Transfer. Biol Reprod. 2009

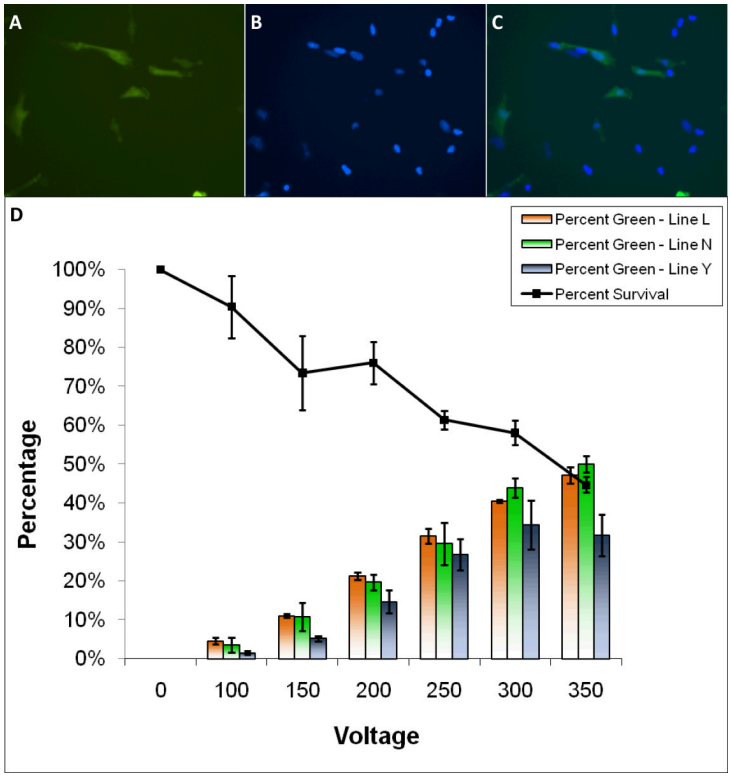

Figure 1.

A–**C**. Representative images demonstrating the ability to use the pCAGG-EGFP vector to identify the number of fibroblast cells that are transiently transfected 24 hours following electroporation. **D**. Across multiple voltages, cell survival was not affected by a cell line x voltage interaction ($P = 0.45$) or by cell line alone ($P = 0.80$), although it was greatly affected by the voltage ($P < 0.001$) cell line and voltage both affected GFP expression ($P < 0.001$ and $P < 0.001$, respectively) while survival at 24 hrs was affected by voltage ($P < 0.001$) and not by cell line ($P = 0.797$). The percent of cells transfected as determined by GFP expression was affected by both cell line ($P < 0.001$) and voltage ($P < 0.001$) although no interaction between the two affects was observed ($P = 0.202$).

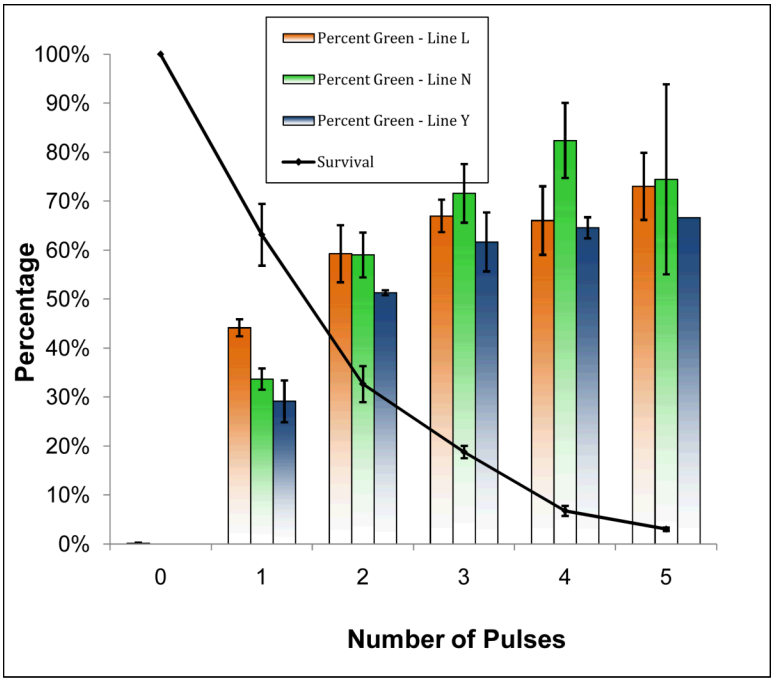

Figure 2.

The percentage of cells being effectively delivered supercoiled vector and expressing GFP was affected by the number of pulses ($P < 0.001$) while cell line had a tendency ($P = 0.06$) to affect the percent green at 300 V. A pulse number x cell line interaction was not detected (P = 0.81). Survival, as depicted by the trendline, was significantly affected by pulse number ($P < 0.001$) while not affected cell line (P < 0.38) or interacting affects of cell line and pulse number ($P = 0.14$).

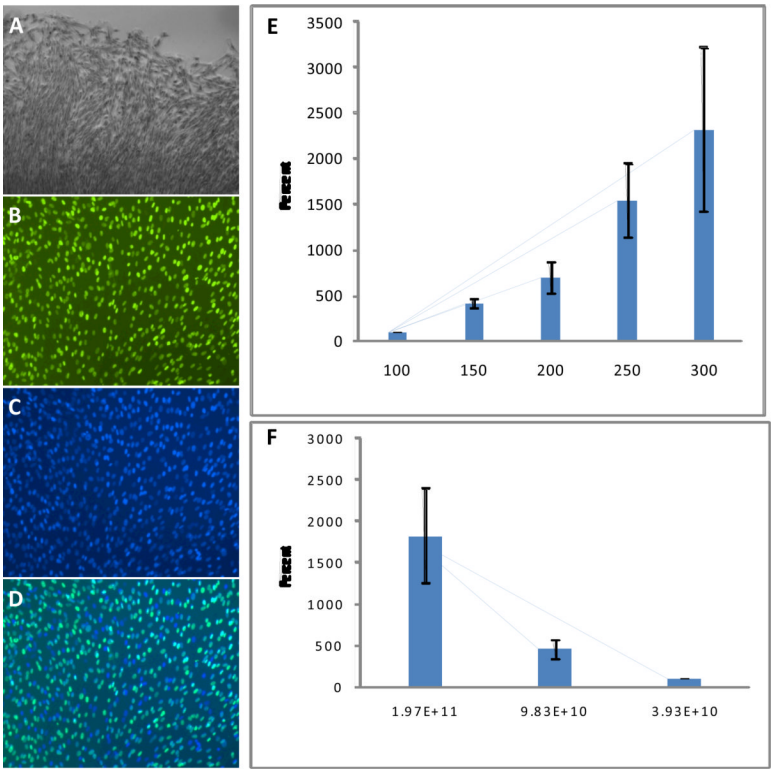

Figure 3.

A. A single G418 resistant colony 14 days following selection using 400 μg/mL G418. **B**–**D**. Representative images demonstrating the variegated expression and nuclear localization of GFP within a single G418 resistant colony 14 days following selection. **E**. The relative ability of different voltages to produce transgenic colonies. G418 resistant colonies produced following selection. Voltage affected ($P < 0.001$) the quantity of G418 resistant colonies produced following electroporation with the 250 and 300 V producing significantly more colonies than 100, 150 or 200 V. **F**. The number of copies of selectable marker, while keeping DNA amount equal, on the number of transgenic colonies produced following electroporation. Copies of selectable marker transgene affected ($P < 0.001$) the number of transgenic colonies produced following electroporation.

Table 1

G418 Resistant Colony Formation.

	Copy Number of Selectable Marker[2]		
Voltage[1]	1.97E+11	9.83E+10	3.93E+10
100	42	14	6
150	181	44	28
200	417	97	22
250	833	264	42
300	1597	333	42

[1] Each voltage was administered with 3 consecutive pulses and was replicated three times, once with each amount of linearized PGK-NEO, the selectable marker gene.

[2] As the copy number of PGK-NEO was reduced, the copies of EF1-GFP was increased maintaining a consistent number of DNA ends.

Table 2

Cloned offspring produced following SCNT using transgenic cells following electroporation and G418 selection.

Cell Line[a]	Number of Reconstructed Embryos Transferred	Embryo Fusion/ Activation Method[b]	Surrogate Number	Outcome	Number Born Alive	Number of Mummified Fetuses	Additional Comments[c]
1GR	69	NT3	O257	Return	N/A	N/A	
6GR	190	NT3	O242	Pregnant	1	4	
1GR	154	NT3	O272	Pregnant	1	3	
Pool 1_GR	174	NT1	O319	Pregnant	4	0	3 died shortly after birth
Pool (20GR, 23GR, 27GR)	190	NT1	O304	Pregnant	2	0	1 died shortly after birth

[a] These cell lines represent G418 resistant clonal cell lines or pools of multiple G418 resistant colonies of fetal fibroblasts.

[b] Electrical and chemical methods of activation correspond to NT1 and NT3, respectively as described (Whitworth et al. 2009).

[c] All cloned piglets that did not die after birth have survived beyond one year.